

All-*trans* retinoic acid down-regulates human albumin gene expression through the induction of C/EBP β -LIP

Takahiro MASAKI*†‡, Tomokazu MATSUURA§, Kiyoshi OHKAWA†, Tatsuo MIYAMURA*, Isao OKAZAKI‡, Tetsu WATANABE‡ and Tetsuro SUZUKI*¹

*Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, †Department of Biochemistry, The Jikei University School of Medicine, Tokyo 105-8461, Japan, ‡Department of Community Health, Tokai University School of Medicine, Kanagawa 259-1193, Japan, and §Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo 105-8461, Japan

ATRA (all-*trans* retinoic acid), which is a major bioactive metabolite of vitamin A and a potent regulator of development and differentiation, mediates down-regulation of the human albumin gene. However, the mechanism of ATRA-mediated down-regulation is not well understood. In the present study, deletion analysis and luciferase assays demonstrate that ATRA causes a marked decrease in the activity of the albumin promoter, the region between nt –367 and –167 from the transcription start site, where C/EBP (CCAAT/enhancer-binding protein)-binding sites are tightly packed, is indispensable for ATRA-mediated down-regulation. ChIP (chromatin immunoprecipitation) assays revealed that *in vivo* binding of C/EBP α to the region markedly decreases upon incubation with ATRA, whereas ATRA treatment marginally increases the recruitment of C/EBP β . We found that ATRA has the ability to differentially and directly induce expression of a truncated isoform of C/EBP β , which is an

LIP (liver-enriched transcriptional inhibitory protein) that lacks a transactivation domain, and to increase the binding activity of C/EBP β -LIP to its response element. Overexpression of C/EBP β -LIP negatively regulates the endogenous expression of albumin, as well as the activity of the albumin promoter induced by C/EBP transactivators such as C/EBP α and full-length C/EBP β . In conclusion, we propose a novel model for down-regulation of the albumin gene, in which ATRA triggers an increase in the translation of C/EBP β -LIP that antagonizes C/EBP transactivators by interacting with their binding sites in the albumin promoter.

Key words: all-*trans* retinoic acid (ATRA), liver-enriched transcription factor, CCAAT/enhancer-binding protein (C/EBP), dominant-negative factor, FLC-4 cell, liver-enriched transcriptional inhibitory protein (LIP).

INTRODUCTION

Serum albumin is the most abundant and characteristic protein that is produced by the mature liver; albumin functions as a transporter of various substances and is a prime regulator of colloid osmotic pressure [1]. Albumin is exclusively synthesized in the liver, approx. 200 mg/kg per day [2], leading to its high steady-state concentration in plasma (35–50 g/l in humans). It has been reported that the albumin level in plasma is decreased as a result of reduced albumin synthesis in clinical disorders such as liver disease [2], infectious disease [3], and cancer [4]. Serum albumin can be used as a reliable indicator for the prognosis and severity of these diseases [5,6]. Therefore it is probable that albumin synthesis is regulated accurately and dramatically in a variety of physiological and pathophysiological conditions.

Albumin synthesis is regulated mainly at the transcriptional level through tissue-specific transcription factors such as HNF (hepatocyte nuclear factor)-1 and C/EBP (CCAAT/enhancer-binding protein) [7,8]. The transcription of the albumin gene is down-regulated by a number of factors, including cytokines [9–11], vitamins [12–14], colloid osmotic pressure [15,16] and amino acid limitation [17]. ATRA (all-*trans* retinoic acid), a major bioactive metabolite of vitamin A, plays a crucial role in hepatocyte differentiation, proliferation and apoptosis [18,19]. ATRA has been shown to down-regulate albumin gene expression in rat hepatocytes [20] and human hepatoma cell lines [12,13].

In animal experiments, it has been reported that a decrease in serum albumin concentration is observed after the administration of ATRA to rodents [21]. Furthermore, in clinical studies of fenretinide (4-hydroxyphenyl-retinamide), a synthetic derivative of ATRA that possesses inhibitory activity against various types of malignant cells [22–24], administration of the drug caused hypoalbuminemia as an adverse effect [25]. Nevertheless, little is known about the ATRA-mediated down-regulation of albumin expression either in experimental or clinical research fields.

In the present study, we have examined the molecular mechanism by which ATRA down-regulates albumin expression in human HCC (hepatocellular carcinoma) cells, with special attention to the transcription factors involved. We present evidence that ATRA preferentially induces the expression of a truncated isoform of C/EBP β : 20 kDa LIP (liver-enriched transcriptional inhibitory protein). We also present evidence that C/EBP β -LIP functions as an antagonist of C/EBP transactivators in the expression of the albumin gene.

EXPERIMENTAL

Plasmids

The promoter fragment of the human albumin gene between nt –1867 and +39 was obtained by PCR amplification using the genomic DNA of human HCC FLC-4 cells [26,27] as a

Abbreviations used: ATRA, all-*trans* retinoic acid; C/EBP, CCAAT/enhancer-binding protein; C/EBP β -FL, C/EBP β -full-length; ChIP, chromatin immunoprecipitation; CUG-BP1, CUG triplet-repeat binding protein 1; DR, direct repeat; eIF, eukaryotic translation initiation factor; EMSA, electrophoretic mobility-shift assay; HCC, hepatocellular carcinoma; HNF, hepatocyte nuclear factor; LAP, liver-enriched transcriptional activator protein; LIP, liver-enriched transcriptional inhibitory protein; RARE, retinoic acid response element; RT, reverse transcriptase.

¹ To whom correspondence should be addressed (email tesuzuki@nih.go.jp).

template; the forward primer (5'-GGCTAGCCTGGACTAATA-TTATTCTTTTCATTTG-3') and the reverse primer (5'-CCTC-GAGGTGTGCCAAAGGCGTGTGGGGT-3') contain restriction sites for *NheI* and *XhoI* respectively at the 5' end. After PCR amplification, the 1.9 kb product was digested with *NheI* and *XhoI* and it was then ligated into the pGL3-Basic vector (Promega, Madison, WI, U.S.A.) to produce pAL1.9-LUC. A series of human albumin promoter 5'-end-deletion constructs were created by PCR amplification using the above reverse primer and the following forward primers: 1456 bp construct (nt - 1417 to +39), 5'-GGCTAGCCAGTACCCATTTCTGAAGAAG-3'; 806 bp construct (nt - 767 to +39), 5'-GGCTAGCCCTCA-TTTGGGTCCATTTTCC-3'; 606 bp construct (nt - 567 to +39), 5'-GGCTAGCCAGCTTTTTCAGACAGAATGG-3'; 406 bp construct (nt - 367 to +39), 5'-GGCTAGCCTATTTAGTTTGG-TTAGTAAT-3' and 206 bp construct (nt - 167 to +39), 5'-GGCTAGCCCAGATGGTAAATATACACAA-3'. Each *NheI/XhoI* fragment was inserted into the pGL3-Basic vector, to yield pAL1.4-LUC, pAL0.8-LUC, pAL0.6-LUC, pAL0.4-LUC or pAL0.2-LUC. To create expression plasmids for C/EBP α , C/EBP β -FL (C/EBP β -full-length) and C/EBP β -LIP, the corresponding sequences were amplified by PCR using pCMV-C/EBP α and pCMV-C/EBP β , which were provided by Dr G. J. Darlington (Department of Pathology, Baylor College of Medicine, Houston, TX, U.S.A.), as templates and primers containing an *EcoRI* site at the 5' ends. Each *EcoRI* fragment was cloned into the *EcoRI* site of the pCAGGS vector [28].

Cell culture and treatment with ATRA

FLC-4 cells were maintained in ASF104 serum-free medium (Ajinomoto, Tokyo, Japan) without any supplements, at 37 °C in a humidified 5 % CO₂/95 % air atmosphere. In experiments using ATRA, FLC-4 cells were cultured in ASF104 serum-free medium with or without 1–1000 nmol/l ATRA (Sigma, St. Louis, MO, U.S.A.). For transient transfection experiments, FLC-4 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10 % foetal bovine serum.

ELISA

Albumin levels in culture medium were measured using a human albumin ELISA quantification kit (Bethyl Laboratories, Montgomery, TX, U.S.A.) according to the manufacturer's instructions.

RNA isolation and real-time RT (reverse transcriptase)-PCR

Total RNA was extracted from FLC-4 cells using the RNeasy mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. Quantitative real-time RT-PCR analysis was performed using the ABI Prism 7700 Sequence Detector (PerkinElmer Applied Biosystems, Foster City, CA, U.S.A.) as described previously [29]. The standard curve was created using serially diluted total RNA obtained from FLC-4 cultures. The amount of target gene expression was calculated from the standard curve, and its quantitative normalization in each sample was carried out using β -actin (PerkinElmer Applied Biosystems) as an internal control. The following primers and fluorescent dual-labelled probes were used: albumin forward primer, 5'-CGATTTTCTT-TTTAGGGCAGTAGC-3'; albumin reverse primer, 5'-TGGAAA-CTTCTGCAAACTCAGC-3'; albumin probe, 5'-CGCCTGAG-CCAGAGATTTCCCA-3'; HNF-1 α forward primer, 5'-AGCG-GGAGGTGGTCGATAC-3'; HNF-1 α reverse primer, 5'-CATG-GGAGTGCCTTGTTG-3'; HNF-1 α probe, 5'-TCAACCAG-

TCCCACCTGTCCCAACA-3'; HNF-1 β forward primer, 5'-AG-CCCAGTTTCCCTTCTATGC-3'; HNF-1 β reverse primer, 5'-TCCTCTTCGGTGGTTTCTTGT-3'; HNF-1 β probe, 5'-CACAATGCCTCTCCCACGATGTCAAG-3'; C/EBP α forward primer, 5'-CAACGTGGAGACGCAGCA-3'; C/EBP α reverse primer, 5'-GCTCAGCTGTTCCACCCG-3'; C/EBP α probe, 5'-CTGACCAGTGACAATGACCGCCTGC-3'; C/EBP β forward primer, 5'-GCCCTCGCAGGTCAAGAG-3'; C/EBP β reverse primer, 5'-TGCGCACGGCGATGT-3' and C/EBP β probe, 5'-CAAGCACAGCGACGAGTACAAGATCCG-3'.

Plasmid transfection and luciferase assay

For the expression of luciferase reporter plasmids in FLC-4 cells, 1×10^5 cells per 22 mm well were seeded and cultured overnight. The adherent cells were transfected with 5 µg of plasmid DNA using TransIT-LT1 transfection reagent (Mirus, Madison, WI, U.S.A.) according to the manufacturer's instructions. After the transfection, the cells were incubated with the fresh medium in the presence or absence of 100 nmol/l ATRA for 48 h, followed by determination of the luciferase activity in cells by using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. For co-expression with C/EBPs, FLC-4 cells were seeded at 2×10^5 cells per 22 mm well on the day before transfection. The cells were transfected with C/EBP expression vectors and the luciferase reporter, pAL1.9-LUC, using TransIT-LT1 transfection reagent, and were cultured for an additional 48 h before the luciferase assay. The total amount of transfected DNA was kept constant by the addition of an empty vector. The pRL-CMV vector (Promega) was used as an internal control for the luciferase assay. To examine the effect of C/EBPs on endogenous albumin expression, FLC-4 cells were seeded at 2×10^5 cells per 22 mm well and cultured overnight. The adherent cells were transfected with C/EBP expression vectors and/or an empty vector using TransIT-LT1 transfection reagent. One day later, the culture medium was changed and the cells were incubated for a further 48 h. After harvesting the medium, the amount of secreted albumin was measured by ELISA.

Western blotting

The proteins were transferred on to a PVDF membrane (Immobilon; Millipore, Bedford, MA, U.S.A.) after separation by SDS/PAGE (12.5 or 15 % gels). After blocking, the membrane was probed with a rabbit polyclonal anti-C/EBP α antibody (sc-61; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), a rabbit polyclonal anti-C/EBP β antibody (sc-150; Santa Cruz Biotechnology), or a mouse monoclonal anti- β -actin antibody (Sigma), followed by incubation with a peroxidase-conjugated secondary antibody and visualization with a SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, U.S.A.). The amount of protein signal was quantified by densitometric analysis (Cool Saver AE-6955; ATTO, Tokyo, Japan).

ChIP (chromatin immunoprecipitation) assay

ChIP assays were performed using the protocol for the ChIP assay kit (Upstate, Lake Placid, NY, U.S.A.). Briefly, FLC-4 cells in 100 mm dishes were grown to 70 % confluency with or without ATRA treatment for 48 h. The chromatin from formaldehyde-fixed FLC-4 cells was sonicated and immunoprecipitated using the rabbit polyclonal anti-C/EBP α or anti-C/EBP β antibodies. The chromatin immunoprecipitate was analysed by PCR (33 cycles) with the following primer pairs (F1/R1 and F2/R2) for two different regions of the albumin gene: the region from nt - 414

to -188 containing four potential binding sites for C/EBP, F1 (5'-GCAATTTGGGACTTAACCTCTTCAGTA-3') and R1 (5'-CCTTGTCATGTATTAAGTTGTGTAACA-3'); and the region from nt -876 to -638 without binding sites for C/EBP, F2 (5'-CAGGGATGGAAAGAATCCTATGCC-3') and R2 (5'-CCATGTTCCCATTCCTGCTGT-3').

EMSA (electrophoretic mobility-shift assay)

Nuclear proteins were extracted from FLC-4 cells that were treated with or without 100 nmol/l ATRA for 48 h, or that transiently expressed the LIP protein, using NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce Biotechnology). A LightShift Chemiluminescent EMSA kit (Pierce Biotechnology) was used to verify the DNA binding of C/EBP β -LIP to potential C/EBP-responsive sequences in the region from nt -414 to -188 of the albumin gene. Nuclear protein (4 μ g) was incubated with the double-stranded biotinylated oligonucleotide that contained the C/EBP-binding site (5'-GTAAATTTGATAAGATGTT-3') for 20 min at room temperature. For competition or supershift assay, a 50-fold molar excess of unlabelled wild-type or mutant oligonucleotides (5'-GTAAACTGATACAATGTT-3'), or 1 μ g of the monoclonal anti-C/EBP β antibody (sc-7962 X; Santa Cruz Biotechnology) was incubated with nuclear extracts for 30 min before the addition of labelled oligonucleotides. Reaction mixtures were then separated on a 6% native polyacrylamide gel, and shifted bands that corresponded to protein-DNA complexes were captured by a horse radish peroxidase-based detection system.

Statistical analysis

Student's *t* test was used to evaluate the statistical difference between groups. All *P* values were obtained using a two-tailed statistical analysis, and *P* < 0.05 was considered statistically significant. Results are means \pm S.D.

RESULTS

Down-regulation of albumin secretion and gene expression by ATRA

Among the established human HCC cell lines, FLC-4 is known to have relatively well-preserved liver cell functions, such as albumin synthesis, enzyme activity and drug metabolism [26,27]. When FLC-4 cells were incubated in the presence of ATRA for 48 h, the albumin level in the culture medium was significantly decreased in a dose-dependent manner; the inhibitory effect of ATRA at concentrations of 1, 10, 100 and 1000 nmol/l on albumin production was $37.04 \pm 3.60\%$, $50.04 \pm 3.27\%$, $59.96 \pm 4.71\%$ and $65.74 \pm 0.74\%$ respectively (Figure 1A). After treatment with 1000 nmol/l ATRA, the growth rate of FLC-4 cells was only slightly (approx. 30%) inhibited, and the cells exhibited no morphological changes (results not shown). To address the mechanism of down-regulation of albumin synthesis by ATRA, the mRNA level of albumin in the cells treated with ATRA for 24 h was examined. As shown in Figure 1(B), consistent with the level of the secreted protein, cellular albumin mRNA expression was inhibited significantly and dose-dependently by ATRA. A 61.95% decrease in the albumin mRNA level was observed in the presence of 100 nmol/l ATRA, demonstrating that albumin synthesis is down-regulated by ATRA via a reduction in the level of mRNA, which is presumably caused by a decrease in transcriptional activity of the albumin gene and/or albumin mRNA stability.

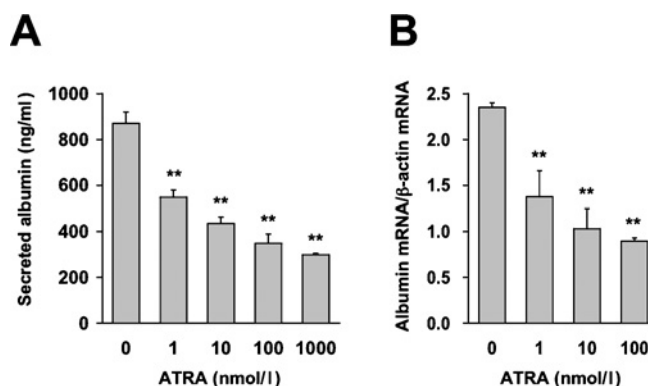


Figure 1 Effect of ATRA treatment on albumin secretion and mRNA expression in FLC-4 cells

(A) The culture medium was collected 48 h after the addition of ATRA. Albumin levels in the medium were quantified by ELISA. ***P* < 0.01 compared with no ATRA treatment. (B) The cells were harvested after incubation with ATRA for 24 h. Albumin mRNA levels were measured by quantitative RT-PCR analysis. Albumin mRNA levels were normalized to β -actin mRNA levels. ***P* < 0.01 compared with control cells without ATRA treatment.

Identification of the 5' end flanking region of the albumin gene responsible for its transcriptional down-regulation by ATRA

It is well known that the effect of ATRA on the transactivation of target gene expression is mediated by retinoic acid receptors and retinoid X-receptors that bind DNA as a heterodimer [30,31]. These nuclear receptors are ligand-dependent transcription factors that bind RAREs (retinoic acid response elements) consisting of two AGGTCA sites usually arranged as DRs (direct repeats) [30,31]. The RAREs found on ATRA-regulated genes are made up of DR motifs with a spacing of 2 (DR2) or 5 nt (DR5) [31]. However, neither the consensus sequence of RAREs nor RARE-like sequences were identified in the 5' end flanking region of the human albumin gene by sequence analysis with a computer search program, TFSEARCH (Searching Transcription Factor Binding Sites; <http://www.cbrc.jp/research/db/TFSEARCH.html>). One can hypothesize that the ATRA-mediated transcriptional regulation of the albumin gene is a secondary response to retinoic acid in human liver cells.

To identify the region responsible for the down-regulation of albumin gene expression by ATRA, a reporter plasmid, pAL1.9-LUC, containing a 1.9 kb fragment of the albumin 5' end flanking sequence linked to a firefly luciferase gene was constructed (Figure 2B) and transfected into FLC-4 cells. The reporter luciferase assay demonstrated that the activity of the albumin promoter from pAL1.9-LUC was markedly decreased to 30.78% after treatment with ATRA (Figure 2B). This inhibitory effect correlated well with the inhibition of albumin mRNA expression (as shown in Figure 1B), suggesting that the 5' end 1.9 kb flanking sequence of the albumin gene is involved in transcriptional down-regulation of albumin expression by ATRA. To define an ATRA-responsive element within the 1.9 kb fragment, a series of 5' end-deletion constructs fused with the luciferase gene were created (Figure 2B). As shown in Figure 2(B), deletions that extended to nt -367 (pAL0.4-LUC) resulted in basal promoter activities and an inhibitory effect of ATRA on promoter activity that are comparable with those observed in the cells transfected with pAL1.9-LUC. By contrast, a further deletion up to nt -167, pAL0.2-LUC, yielded a marked decrease in the basal promoter activity, as well as in the inhibitory effect of ATRA on the promoter activity. These results suggest that the region from nt -367 to -167 contains a putative element responsible for

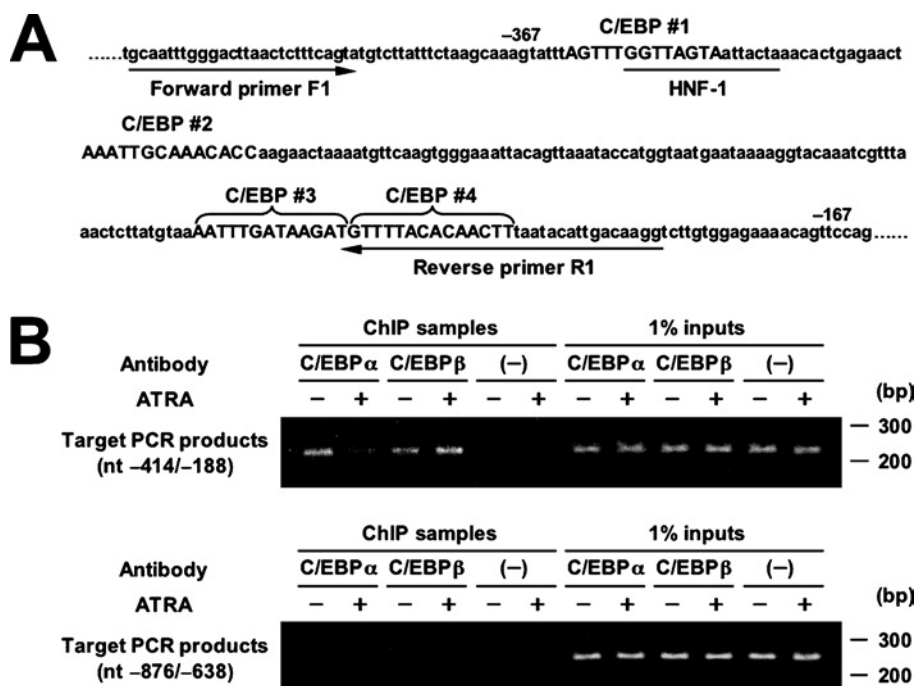


Figure 4 Effects of ATRA on *in vivo* recruitment of C/EBPs to the human albumin promoter

(A) The sequence of the upstream regulatory region (between nt -414 and -188) of human albumin was amplified using a PCR primer pair (F1 and R1), indicated by the arrows. The potential binding sites for C/EBP and HNF-1 in this region are in capital letters and underlined respectively. (B) FLC-4 cells were incubated with or without ATRA (100 nmol/l) for 48 h before the ChIP assay. DNA fragments were amplified with the primers F1 and R1 (upper panel) or F2 and R2 (lower panel). The input represents PCR products from chromatin pellets before immunoprecipitation.

the albumin promoter *in vivo*, we performed ChIP assays of cells treated with or without ATRA (Figure 4). After immunoprecipitation with antibodies against C/EBP α and C/EBP β , the -414 to -188 nt fragment within the albumin promoter, which contains four potential binding sites for C/EBPs (C/EBP #1–#4 in Figure 4A), was amplified by PCR. The results revealed that under basal conditions both endogenous C/EBP α and C/EBP β were recruited to the albumin promoter. It is of interest that the *in vivo* binding of C/EBP α to the fragment was markedly decreased upon incubation with ATRA. By contrast, ATRA treatment seemed to marginally increase the recruitment of C/EBP β to the promoter fragment (Figure 4B). We verified that no DNA fragment was detected in the precipitated chromatin when the region between nt -876 and -638 of the albumin gene, which does not contain binding sites for C/EBP, was amplified by PCR.

These findings suggest that ATRA has the ability to affect the C/EBP occupancy of the albumin promoter *in vivo*. It is possible that impaired C/EBP α binding caused by ATRA leads to down-regulation of albumin gene expression.

ATRA induces expression of C/EBP β -LIP, and its DNA-binding activity

It has been reported that the gene for C/EBP is transcribed into a single mRNA that encodes several N-terminally truncated protein isoforms, possibly via the process of alternative translation initiation at downstream AUG codons [34,35]. C/EBP α mRNA is translated into two major proteins of 42 and 30 kDa (p42- and p30-C/EBP α) [34,36], whereas C/EBP β mRNA mainly produces three isoforms referred to as C/EBP β -FL, LAP (liver-enriched transcriptional activator protein) and LIP, which are 46, 42, and 20 kDa respectively [34,37,38]. All of the C/EBP isoforms have DNA-binding and dimerization domains. However, p30-C/EBP α

and LIP are translated from the third in-frame AUG start codon [34,36] and lack most of the transactivation domain [34,37,39]. Although p42-C/EBP α , C/EBP β -FL and LAP transactivate their target genes' expression, p30-C/EBP α and LIP are unable to activate gene transcription and are able to function as dominant-negative factors by antagonizing and by competing with other C/EBP transactivators [34,37]. It has been considered that the ratio of C/EBP transactivators to C/EBP dominant-negative factors is important in controlling each activity of C/EBP α and C/EBP β [34,36,37].

We determined the effect of ATRA on the expression of C/EBP isoforms by Western blotting (Figure 5A). An increased level of LIP was observed as early as 8 h after ATRA treatment and it was also detected after 48 h of treatment. By contrast, C/EBP β -FL and LAP isoforms showed little change in their expression after the 48 h time point of ATRA treatment. The expression level of C/EBP α showed no change in the presence of ATRA after 8 or 16 h. Although a decrease in C/EBP α expression was found after 48 h of ATRA treatment, it is not likely that this reduction is the cause of down-regulation of albumin expression, since a decreased level of albumin mRNA was observed as early as 24 h after ATRA treatment (Figure 1B). It appears that the ratio of LIP to C/EBP β -FL and LAP was elevated in a dose-dependent manner after 16 h of ATRA treatment, while the ratio of p42-C/EBP α to p30-C/EBP α showed no difference with or without ATRA treatment until 48 h after the addition of ATRA. These results demonstrate that ATRA has the ability to differentially modulate the level of C/EBP isoforms with an increase in LIP and a decrease in C/EBP α ; the expression of LIP is induced immediately in response to ATRA treatment.

We next examined the effect of ATRA on the DNA-binding activity of LIP, by EMSA (Figure 5B). The nucleotide sequence of C/EBP-binding site #3, which is present in the region from

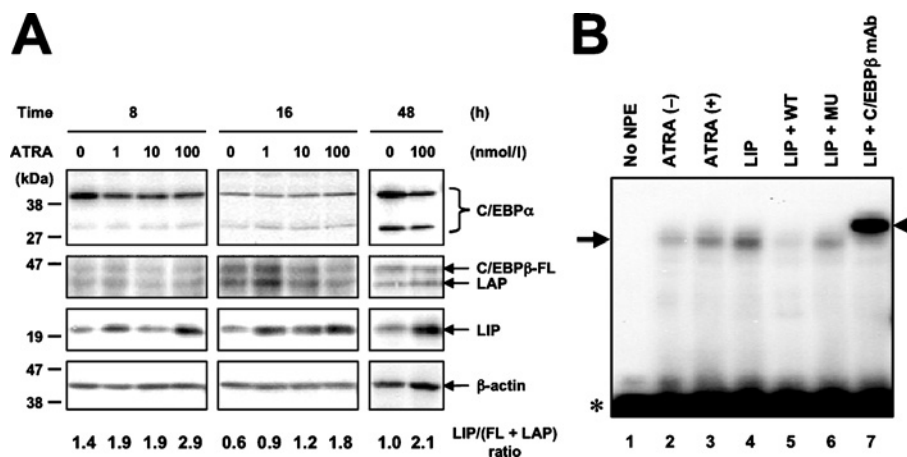


Figure 5 Effect of ATRA on protein expression of C/EBPs, and DNA-binding activity of C/EBPβ-LIP

(A) FLC-4 cells were incubated with ATRA (100 nmol/l) for 8, 16 and 48 h. After harvesting, total cellular proteins were resolved by SDS/PAGE, and the presence of C/EBPα and C/EBPβ was analysed by Western blotting. The ratio of C/EBPβ-LIP to C/EBPβ-FL and -LAP [LIP/(FL + LAP)] was measured by densitometric analysis. (B) Nuclear proteins were extracted from FLC-4 cells treated with or without 100 nmol/l ATRA for 48 h, or cells that transiently expressed the LIP protein, and were analysed by EMSA as described in the Experimental section. Lane 1, no nuclear protein extracts (NPE); lane 2, FLC-4 cells incubated without ATRA; lane 3, FLC-4 cells incubated with ATRA; lane 4, FLC-4 cells transiently expressing the LIP protein; lane 5, addition of a 50-fold molar excess of unlabelled wild-type (WT) oligonucleotides to the mixture in lane 4; lane 6, addition of a 50-fold molar excess of unlabelled mutant (MU) oligonucleotides to the mixture in lane 4; lane 7, addition of an anti-C/EBPβ monoclonal antibody (mAb) to the mixture in lane 4. The arrow, arrowhead and asterisk indicate a DNA-LIP complex, a DNA-LIP-antibody complex (supershifted band) and a free probe respectively.

nt -367 to -167 of the albumin gene, as shown in Figure 4(A), was used as a DNA probe. A shifted band, which was observed in FLC-4 cells that transiently express LIP protein (Figure 5, lane 4), was removed by the addition of an excess of unlabelled homologous probe (lane 5), but not by the addition of a mutated sequence (lane 6). This band was supershifted by the addition of the C/EBPβ antibody (lane 7). The results indicate that LIP binds to its binding motif within the albumin promoter.

In the presence of ATRA (Figure 5, lane 3), a shifted band that corresponded to endogenous LIP-bound DNA was observed, which was more intense than in the absence of ATRA (lane 2). We also confirmed that endogenous LIP bound to the other three binding sites for C/EBP (#1, #2 and #4) (results not shown). Thus the combined data demonstrate that ATRA induces not only the expression of LIP but also its DNA-binding activity.

C/EBPβ-LIP down-regulates the gene expression and synthesis of albumin by blocking the transcriptional activity of C/EBPα and C/EBPβ-FL

Our data suggest that early induction of LIP expression and an increase in the DNA-binding activity of LIP caused by ATRA triggers the down-regulation of albumin gene expression. To directly evaluate the role of LIP in albumin expression, we determined the effect of C/EBPs on albumin expression using transient transfection experiments (Figure 6). As shown in Figure 6(A), overexpression of C/EBPα and C/EBPβ-FL caused a more than 20.7- or 8-fold increase respectively, in promoter activity in a dose-dependent manner compared with transfection with an empty vector. By contrast, co-expression with LIP resulted in a marked and dose-dependent reduction of the increased activity (Figure 6B). Expression of LIP alone also decreased the level of promoter activity (Figure 6B), presumably because LIP inhibited positive functions of endogenous C/EBP transactivators expressed in FLC-4 cells. We investigated the effect of C/EBP expression on the secretion of endogenous albumin (Figure 6C). Consistent with albumin promoter activity, overexpression of C/EBPα and C/EBPβ-FL significantly increased the level of

albumin in the culture medium by 2- and 1.7-fold respectively, compared with transfection with an empty vector. Overexpression of LIP significantly and dose-dependently decreased both the basal level of albumin secretion and the level of albumin elevated by expression of C/EBPα and C/EBPβ-FL. Thus these results strongly suggest that LIP plays a role in repressing albumin gene expression by blocking the ability of C/EBP transactivators to activate the albumin promoter, leading to down-regulation of albumin synthesis.

DISCUSSION

In the present study, we have demonstrated the down-regulation of secretion and gene expression of albumin mediated by ATRA. This finding is in line with previous reports that ATRA negatively regulates albumin synthesis in rat hepatocytes [20] and human hepatoma cell lines [12,13]. We addressed its molecular mechanisms and found: (i) that the 5' end flanking region of the albumin gene, nt -367 to -167, in which four binding sites for C/EBPs are conserved, is responsible for its transcriptional repression by ATRA, and (ii) that upon ATRA treatment, C/EBPβ-LIP is differentially induced among C/EBPβ isoforms and the expression of C/EBPα is subsequently decreased. The present study reveals that C/EBP proteins play a key role in the transcriptional regulation of the human albumin gene by ATRA.

C/EBPα and C/EBPβ, which are composed of DNA-binding and dimerization domains at their C-termini, and transactivation domains at their N-terminal regions, are expressed in the liver at high levels and are involved in the regulation of cell growth, cell differentiation, metabolism and inflammation [40-42]. A single mRNA species for C/EBPβ directs production of three major isoforms in liver tissues: C/EBPβ-FL, LAP and a low-molecular-mass isoform, LIP [34,37,38]. These three proteins contain the DNA-binding and dimerization domains. C/EBPβ-FL and LAP contain a transactivation domain and function as transcriptional activators, whereas LIP lacks the N-terminal transactivation domain and can attenuate the transcriptional stimulation by

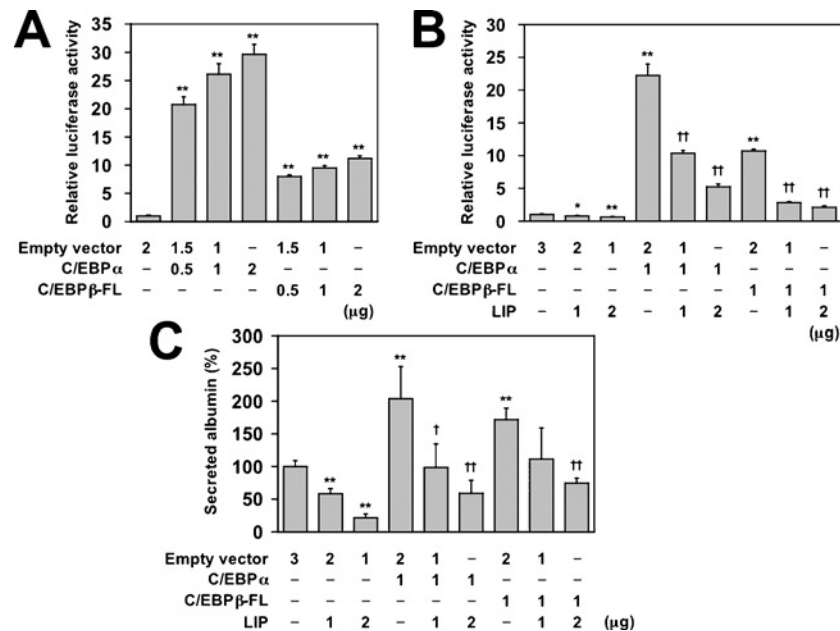


Figure 6 C/EBP β -LIP down-regulates the promoter activity and synthesis of albumin by blocking C/EBP transcriptional activity

(A) and (B) FLC-4 cells were transfected with pAL1.9-LUC and pRL-CMV in the presence or absence of C/EBP expression vectors. After incubation for 48 h, the cells were harvested and assayed for luciferase activity. The relative luciferase activity was obtained by normalizing the pAL1.9-LUC activity to the pRL-CMV activity, and the value of the empty vector (pCAGGS) was set to 1. * P < 0.05; ** P < 0.01 compared with cells transfected with empty vector. †† P < 0.01 compared with cells expressing C/EBP α or C/EBP β -FL alone. (C) FLC-4 cells were transfected with C/EBP expression vectors and/or an empty vector. After 72 h, the medium was harvested, and the amount of secreted albumin was quantified by ELISA. The value of the empty vector was set to 100%. ** P < 0.01 compared with transfection with empty vector. † P < 0.05; †† P < 0.01 compared with cultures that express C/EBP α or C/EBP β -FL alone.

C/EBP β -FL, LAP and C/EBP α in substoichiometric amounts [37]. It has been shown that LIP is involved in the down-regulation of human *CYP3A4* induced by interleukin-6 [43] and in the repression of C/EBP α mRNA during the acute phase of the immune response [44]. In the present study, we have demonstrated that the overexpression of LIP leads to inhibition of the positive regulation of albumin promoter activity that is mediated by C/EBP α and C/EBP β -FL (Figure 6B). This is the first study to show the involvement of LIP in the regulation of human albumin gene expression.

Our albumin promoter assay using the 5' end-deletion constructs demonstrated that the region spanning nt -367 to -167 within the albumin gene is a prerequisite for ATRA-dependent transcriptional down-regulation (Figure 2B). The ChIP assay showed that during ATRA treatment the *in vivo* binding of C/EBP α to the region clearly decreased, whereas the binding of C/EBP β slightly increased (Figure 4B). The anti-C/EBP β antibody used in the present study is capable of recognizing all of the C/EBP β isoforms, since no anti-C/EBP β antibodies that recognize the N-termini of C/EBP β are available to distinguish these isoforms. However, an EMSA demonstrated that ATRA increased the binding activity of LIP to its response element in this region (Figure 5B). Thus we suggest that the increase in DNA-binding of C/EBP β , as shown in the ChIP assay, is caused by ATRA-induced LIP expression. It has been reported that LIP has a greater binding capacity for the C/EBP-binding element compared with C/EBP transactivators i.e. LIP binds to the element 4-fold more efficiently than does LAP [37]. Therefore it is probable that C/EBP transactivators predominantly bind to their response elements within the albumin promoter in the ATRA-untreated cells, while an increased level of LIP possibly displaces these transactivators at the C/EBP-binding sites to occupy them in ATRA-treated cells.

As indicated in Figure 5(A), preferential induction of LIP expression by ATRA was observed even in the culture after

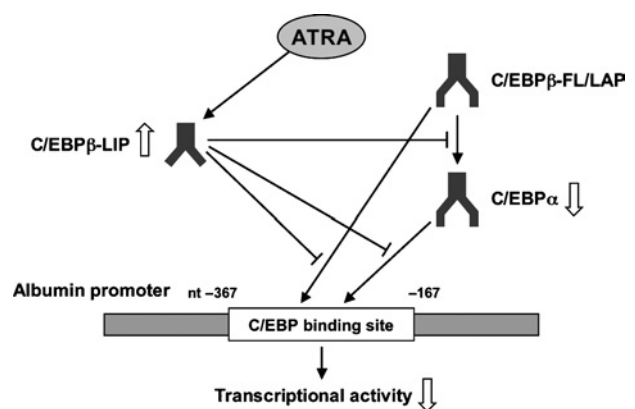


Figure 7 Proposed mechanism by which ATRA down-regulates albumin gene transcription in FLC-4 cells

An ATRA signal increases the expression of C/EBP β -LIP in FLC-4 cells. This truncated C/EBP protein directly and indirectly down-regulates the expression of the albumin gene by competing with C/EBP transactivators and inhibiting C/EBP α expression.

8 h of ATRA treatment. By contrast, a decrease in C/EBP α gene expression was not detected at such an early stage (results not shown), but was first observed 24 h after the addition of ATRA (Figure 3C). Furthermore, the expression level of C/EBP α protein was little changed when the mRNA expression level of albumin was decreased by ATRA treatment (Figure 5A). Based on the results of the present study, we propose a model for the molecular mechanism by which ATRA down-regulates the expression of human albumin in liver-derived cells (Figure 7). ATRA triggers the differential induction of C/EBP β -LIP, a dominant-negative regulator of C/EBP activators. A C/EBP-binding consensus sequence has been identified in the C/EBP α

promoter, and it has been shown that C/EBP β -FL and LIP respectively, function as positive and negative regulators of C/EBP α gene expression [44]. Thus it is probable that LIP preferentially binds to the C/EBP-binding elements not only on the albumin promoter but also on that of C/EBP α at the expense of C/EBP β -FL, LAP and C/EBP α . However, LIP lacks the transactivation domain and is unable to activate transcription. LIP expression induced by ATRA possibly down-regulates the gene expression of albumin and C/EBP α , by competing for DNA-binding sites as a LIP homodimer and/or by antagonizing the transcriptional activity of C/EBP transactivators via heterodimer formation with C/EBP-FL or LAP [37]. Hence, an increase in the ratio of LIP to C/EBP transactivators is critical for down-regulation of the expression of albumin and C/EBP α genes that is mediated by ATRA. This conclusion is supported by transient transfection experiments which indicated that the activity of the albumin promoter is stimulated by transfection with C/EBP β -FL or C/EBP α expression constructs (Figure 6A), whereas its activity is decreased by co-transfection with a LIP construct (Figure 6B). Decreased expression of C/EBP α may also contribute to the transcriptional repression of the albumin gene, since C/EBP α is known to be one of the positive regulators of albumin expression [32,33].

A key question is: how does ATRA differentially induce C/EBP β -LIP but not C/EBP β -FL expression? A previous study [45], which describes the effect of ATRA on the alternate production of C/EBP β isoforms, has not demonstrated its molecular mechanism. A number of recent observations have shown that the production of C/EBP β isoforms is regulated by epidermal growth factor [38], lipopolysaccharide [44,46] and partial hepatectomy [44,47], presumably through leaky ribosomal scanning [34,37]. It has been proposed that a portion of ribosomes ignore the first two AUG codons of the C/EBP β mRNA and initiate translation of LIP from the third in-frame AUG start codon. The translation of LIP can be controlled by specific cytoplasmic proteins that interact with the 5' end region of C/EBP β mRNA, such as CUG-BP1 (CUG triplet-repeat binding protein 1) [44,47]. Phosphorylation of CUG-BP1 is critical for its RNA binding and the consequent increase in LIP expression [38]. Therefore we tested whether ATRA treatment leads to increased phosphorylation of CUG-BP1 in FLC-4 cells. Western blotting and immunoprecipitation of CUG-BP1 metabolically labelled with 32 P, however, indicated that the expression level and phosphorylation status of CUG-BP1 were not different in cells with or without ATRA treatment (results not shown). It can be speculated that other RNA-binding proteins are involved in the mechanism by which ATRA increases the translation of LIP because, in addition to CUG-BP1, calreticulin [48] and eIFs (eukaryotic translation initiation factors), such as eIF-2 and eIF-4E [35], have been shown to control translation of C/EBP β . Another possible mechanism for the alternate production of C/EBP β isoforms is the proteolytic cleavage of C/EBP β -FL [49]. However, this is less likely to explain the mechanism for the differential induction of LIP isoforms caused by ATRA, because it has been shown that the cleavage of C/EBP β -FL to generate LIP is induced by C/EBP α [49], but that the ATRA treatment led to no change or only a small decrease in the expression of C/EBP α (Figure 5A).

A previous study [13] has shown that the expression of HNF-1, which is a potent transcription factor for the albumin gene [50], was decreased in human hepatoma cells in which albumin gene expression was down-regulated by ATRA, although an upstream regulatory region of the albumin gene involved in the regulation was not identified. In our experimental setting, mRNA expression of HNF-1 was not affected by ATRA treatment (Figures 3A and

3B). The data in the present study suggest a model in which the preferential increase in LIP expression mediated by ATRA results in the antagonization of C/EBP transactivators by interaction with their binding sites on the nt -367 to -167 region of the albumin promoter. We propose a novel pathway for the modulation of gene expression by ATRA, in which C/EBP β -LIP plays a crucial role. This mechanism may be found in other systems for physiological processes, such as cell proliferation and differentiation, and the elucidation of its molecular mechanism will make a great contribution to our understanding of gene regulation mediated by retinoic acids.

We thank G. J. Darlington for providing the plasmids, and A. Catharine Ross (Department of Nutritional Sciences, The Pennsylvania State University) and our colleagues for helpful discussions. We also thank M. Matsuda, M. Ikeda, S. Yoshizaki and T. Shimoji for their technical assistance. This work was supported by grants-in-aid from the Ministry of Health, Labor and Welfare, by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan (ID:01-3), and by Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, Japan. T. M. is the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research in Japan.

REFERENCES

- West, J. B. (1990) Blood and the plasma proteins: functions and composition of blood. In *Physiological Basis of Medical Practice*. In (West, J. B., ed.), pp. 332–338, Williams & Wilkins, Baltimore.
- Rothschild, M. A., Oratz, M. and Schreiber, S. S. (1988) Serum albumin. *Hepatology* **8**, 385–401.
- Moshage, H. J., Janssen, J. A., Franssen, J. H., Hafkenscheid, J. C. and Yap, S. H. (1987) Study of the molecular mechanism of decreased liver synthesis of albumin in inflammation. *J. Clin. Invest.* **79**, 1635–1641.
- Mariani, G., Strober, W., Keiser, H. and Waldmann, T. A. (1976) Pathophysiology of hypoalbuminemia associated with carcinoid tumor. *Cancer* **38**, 854–860.
- Chlebowski, R. T., Grosvenor, M. B., Bernhard, N. H., Morales, L. S. and Bulcavage, L. M. (1989) Nutritional status, gastrointestinal dysfunction, and survival in patients with AIDS. *Am. J. Gastroenterol.* **84**, 1288–1293.
- Phillips, A., Shaper, A. G. and Whincup, P. H. (1989) Association between serum albumin and mortality from cardiovascular disease, cancer, and other causes. *Lancet* **2**, 1434–1436.
- Gorski, K., Carneiro, M. and Schibler, U. (1986) Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell* **47**, 767–776.
- Maire, P., Wuarin, J. and Schibler, U. (1989) The role of *cis*-acting promoter elements in tissue-specific albumin gene expression. *Science* **244**, 343–346.
- Perlmutter, D. H., Dinarello, C. A., Punsal, P. I. and Colten, H. R. (1986) Cachectin/tumor necrosis factor regulates hepatic acute-phase gene expression. *J. Clin. Invest.* **78**, 1349–1354.
- Andus, T., Geiger, T., Hirano, T., Kishimoto, T. and Heinrich, P. C. (1988) Action of recombinant human interleukin 6, interleukin 1 β and tumor necrosis factor α on the mRNA induction of acute-phase proteins. *Eur. J. Immunol.* **18**, 739–746.
- Morrone, G., Cortese, R. and Sorrentino, V. (1989) Post-transcriptional control of negative acute phase genes by transforming growth factor β . *EMBO J.* **8**, 3767–3771.
- Hsu, S. L., Lin, Y. F. and Chou, C. K. (1992) Transcriptional regulation of transferrin and albumin genes by retinoic acid in human hepatoma cell line Hep3B. *Biochem. J.* **283**, 611–615.
- Yamada, Y., Shidoji, Y., Fukutomi, Y., Ishikawa, T., Kaneko, T., Nakagama, H., Imawari, M., Moriaki, H. and Muto, Y. (1994) Positive and negative regulations of albumin gene expression by retinoids in human hepatoma cell lines. *Mol. Carcinog.* **10**, 151–158.
- Molina, A., Oka, T., Muñoz, S. M., Chikamori-Aoyama, M., Kuwahata, M. and Natori, Y. (1997) Vitamin B $_6$ suppresses growth and expression of albumin gene in a human hepatoma cell line HepG2. *Nutr. Cancer* **28**, 206–211.
- Tsutsumi, T., Nakao, K., Mitsuoka, S., Hamasaki, K., Tsuruta, S., Shima, M., Nakata, K., Tamaoki, T. and Nagataki, S. (1993) Regulation of albumin and α -fetoprotein gene expression by colloid osmotic pressure in human hepatoma cells. *Gastroenterology* **104**, 256–262.
- Pietrangolo, A. and Shafritz, D. A. (1994) Homeostatic regulation of hepatocyte nuclear transcription factor 1 expression in cultured hepatoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 182–186.
- Marten, N. W., Hsiang, C. H., Yu, L., Stollenwerk, N. S. and Straus, D. S. (1999) Functional activity of hepatocyte nuclear factor-1 is specifically decreased in amino acid-limited hepatoma cells. *Biochim. Biophys. Acta* **1447**, 160–174.

- 18 Falasca, L., Favale, A., Gualandi, G., Maietta, G. and Conti Devirgiliis, L. (1998) Retinoic acid treatment induces apoptosis or expression of a more differentiated phenotype on different fractions of cultured fetal rat hepatocytes. *Hepatology* **28**, 727–737
- 19 Alisi, A., Leoni, S., Piacentani, A. and Conti Devirgiliis, L. (2003) Retinoic acid modulates the cell-cycle in fetal rat hepatocytes and HepG2 cells by regulating cyclin-cdk activities. *Liver Int.* **23**, 179–186
- 20 Ikeda, H. and Fujiwara, K. (1993) Retinoic acid inhibits DNA and albumin synthesis stimulated by growth factor in adult rat hepatocytes in primary culture. *Biochem. Biophys. Res. Commun.* **191**, 675–680
- 21 Sani, B. P. and Meeks, R. G. (1983) Subacute toxicity of all-*trans*- and 13-*cis*-isomers of *N*-ethyl retinamide, *N*-2-hydroxyethyl retinamide, and *N*-4-hydroxyphenyl retinamide. *Toxicol. Appl. Pharmacol.* **70**, 228–235
- 22 Decensi, A., Bruno, S., Costantini, M., Torrisi, R., Curotto, A., Gatteschi, B., Nicolò, G., Polizzi, A., Perloff, M., Malone, W. F. and Bruzzi, P. (1994) Phase IIa study of fenretinide in superficial bladder cancer, using DNA flow cytometry as an intermediate end point. *J. Natl. Cancer Inst.* **86**, 138–140
- 23 Kalemkerian, G. P., Slusher, R., Ramalingam, S., Gadgeel, S. and Mabry, M. (1995) Growth inhibition and induction of apoptosis by fenretinide in small-cell lung cancer cell lines. *J. Natl. Cancer Inst.* **87**, 1674–1680
- 24 Maurer, B. J., Metelitsa, L. S., Seeger, R. C., Cabot, M. C. and Reynolds, C. P. (1999) Increase of ceramide and induction of mixed apoptosis/necrosis by *N*-(4-hydroxyphenyl)-retinamide in neuroblastoma cell lines. *J. Natl. Cancer Inst.* **91**, 1138–1146
- 25 Puduvalli, V. K., Yung, W. K., Hess, K. R., Kuhn, J. G., Groves, M. D., Levin, V. A., Zwiebel, J., Chang, S. M., Cloughesy, T. F., Junck, L. et al. (2004) Phase II study of fenretinide (NSC 374551) in adults with recurrent malignant gliomas: a North American brain tumor consortium study. *J. Clin. Oncol.* **22**, 4282–4289
- 26 Nagamori, S., Hasumura, S., Matsuura, T., Aizaki, H. and Kawada, M. (2000) Developments in bioartificial liver research: concepts, performance, and applications. *J. Gastroenterol.* **35**, 493–503
- 27 Aizaki, H., Nagamori, S., Matsuda, M., Kawakami, H., Hashimoto, O., Ishiko, H., Kawada, M., Matsuura, T., Hasumura, S., Matsuura, Y. et al. (2003) Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor. *Virology* **314**, 16–25
- 28 Niwa, H., Yamamura, K. and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199
- 29 Iwahori, T., Matsuura, T., Maehashi, H., Sugo, K., Saito, M., Hosokawa, M., Chiba, K., Masaki, T., Aizaki, H., Ohkawa, K. and Suzuki, T. (2003) CYP3A4 inducible model for *in vitro* analysis of human drug metabolism using a bioartificial liver. *Hepatology* **37**, 665–673
- 30 Mangelsdorf, D. J. and Evans, R. M. (1995) The RXR heterodimers and orphan receptors. *Cell* **83**, 841–850
- 31 Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10**, 940–954
- 32 Friedman, A. D., Landschulz, W. H. and McKnight, S. L. (1989) CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. *Genes Dev.* **3**, 1314–1322
- 33 Trautwein, C., Rakemann, T., Pietrangelo, A., Plümpe, J., Montosi, G. and Manns, M. P. (1996) C/EBP- β /LAP controls down-regulation of albumin gene transcription during liver regeneration. *J. Biol. Chem.* **271**, 22262–22270
- 34 Ossipow, V., Descombes, P. and Schibler, U. (1993) CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8219–8223
- 35 Calkhoven, C. F., Müller, C. and Leutz, A. (2000) Translational control of C/EBP α and C/EBP β isoform expression. *Genes Dev.* **14**, 1920–1932
- 36 Lin, F. T., MacDougald, O. A., Diehl, A. M. and Lane, M. D. (1993) A 30-kDa alternative translation product of the CCAAT/enhancer binding protein α message: transcriptional activator lacking antimitotic activity. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9606–9610
- 37 Descombes, P. and Schibler, U. (1991) A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* **67**, 569–579
- 38 Baldwin, B. R., Timchenko, N. A. and Zahnow, C. A. (2004) Epidermal growth factor receptor stimulation activates the RNA binding protein CUG-BP1 and increases expression of C/EBP β -LIP in mammary epithelial cells. *Mol. Cell. Biol.* **24**, 3682–3691
- 39 Calkhoven, C. F., Bouwman, P. R., Snippe, L. and Ab, G. (1994) Translation start site multiplicity of the CCAAT/enhancer binding protein α mRNA is dictated by a small 5' open reading frame. *Nucleic Acids Res.* **22**, 5540–5547
- 40 Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R. and Darlington, G. J. (1995) Impaired energy homeostasis in C/EBP α knockout mice. *Science* **269**, 1108–1112
- 41 Poli, V. (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J. Biol. Chem.* **273**, 29279–29282
- 42 Wang, H., Iakova, P., Wilde, M., Welm, A., Goode, T., Roesler, W. J. and Timchenko, N. A. (2001) C/EBP α arrests cell proliferation through direct inhibition of Cdk2 and Cdk4. *Mol. Cell* **8**, 817–828
- 43 Jover, R., Bort, R., Gómez-Lechón, M. J. and Castell, J. V. (2002) Down-regulation of human CYP3A4 by the inflammatory signal interleukin 6: molecular mechanism and transcription factors involved. *FASEB J.* **16**, 1799–1801
- 44 Welm, A. L., Mackey, S. L., Timchenko, L. T., Darlington, G. J. and Timchenko, N. A. (2000) Translational induction of liver-enriched transcriptional inhibitory protein during acute phase response leads to repression of CCAAT/enhancer binding protein α mRNA. *J. Biol. Chem.* **275**, 27406–27413
- 45 Hsu, W. and Chen-Kiang, S. (1993) Convergent regulation of NF-IL6 and Oct-1 synthesis by interleukin-6 and retinoic acid signaling in embryonal carcinoma cells. *Mol. Cell. Biol.* **13**, 2515–2523
- 46 Hsieh, C. C., Xiong, W., Xie, Q., Rabek, J. P., Scott, S. G., An, M. R., Reisner, P. D., Kuninger, D. T. and Papaconstantinou, J. (1998) Effects of age on the posttranscriptional regulation of CCAAT/enhancer binding protein α and CCAAT/enhancer binding protein β isoform synthesis in control and LPS-treated livers. *Mol. Biol. Cell* **9**, 1479–1494
- 47 Timchenko, N. A., Welm, A. L., Lu, X. and Timchenko, L. T. (1999) CUG repeat binding protein (CUGBP1) interacts with the 5' region of C/EBP β mRNA and regulates translation of C/EBP β isoforms. *Nucleic Acids Res.* **27**, 4517–4525
- 48 Timchenko, L. T., Iakova, P., Welm, A. L., Cai, Z. J. and Timchenko, N. A. (2002) Calreticulin interacts with C/EBP α and C/EBP β mRNAs and represses translation of C/EBP proteins. *Mol. Cell. Biol.* **22**, 7242–7257
- 49 Welm, A. L., Timchenko, N. A. and Darlington, G. J. (1999) C/EBP α regulates generation of C/EBP β isoforms through activation of specific proteolytic cleavage. *Mol. Cell. Biol.* **19**, 1695–1704
- 50 Lichtsteiner, S. and Schibler, U. (1989) A glycosylated liver-specific transcription factor stimulates transcription of the albumin gene. *Cell* **57**, 1179–1187

Received 21 November 2005/20 March 2006; accepted 11 April 2006

Published as BJ Immediate Publication 11 April 2006, doi:10.1042/BJ20051863